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Biodegradation and biosorption for decolorization of synthetic dyes by *Funalia trogii*

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Abstract

The objective of this study was to investigate two major mechanisms for decolorization of dyes by cultivation of fungi on either a solid or in a liquid phase and to confirm the possibility of practical application via repeated-batch cultivation. The decolorization of six commercial dyes with 10 fungal strains was studied. Enzyme activity, decolorization trends and decolorization mechanisms were monitored. Under the experimental conditions, extracellular laccase and manganese peroxidase (MnP), but not lignin peroxidase (LiP), were detected. The decolorization mechanisms by *F. trogii* ATCC 200800 involved a complex interaction of enzyme activity and biosorption. This study suggests that it is possible to decolorize a high concentration of commercial dyes, which would be a great advance in the treatment of dye containing wastewater. These methods may have a potential application for dye decolorization and for textile effluent treatment.
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1. Introduction

Large amounts of chemically different dyes are used for various industrial applications such as textile dyeing, and a significant proportion of these dyes enter the environment in wastewater. The presence of very low concentrations of dyes in effluent can be highly visible and undesirable [1–3]. There are more than 10^5 kinds of commercially available dyes with over 7×10^5 tonnes of dyestuff produced annually. These dyes are designed to be resistant to light, water and oxidizing agents and are therefore difficult to degrade once released into aquatic systems [4,5].

The treatment of dye wastewater involves chemical and physical methods such as adsorption, coagulation, oxidation, filtration and ionizing radiation. All these methods have different decolorization capabilities, capital costs and operating speed. Among these methods, coagulation and adsorption are the commonly used, however these create huge amounts of sludge which become a pollutant creating its own creating disposal problems. There is a great need to develop an economic and effective way of dealing with the textile dyeing waste in the face of the ever increasing production activities. Biological processes have received increasing interest as a viable alternative owing to their cost effectiveness, ability to produce less sludge and environmental friendliness [6–9]. Synthetic dyes containing various substituents such as nitro and sulfonic groups are not uniformly susceptible to biodecolorization in conventional aerobic processes. Attempts to develop aerobic bacterial strains for dye decolorization often resulted in very specific organisms which showed decolorization capability for individual dyes [10]. Our recent studies also indicated that those results form a basis for developing more efficient treatment system for dye and textile effluents to achieve decolorization and degradation of residual dyes [1,9,11,12].

Over the past decade, many fungal strains have been studied for their abilities to degrade a wide variety of structurally diverse

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pollutants. Recently, many studies have also demonstrated that fungi are able to degrade dyes and this capability to degrade dye is due to the extracellular, nonspecific and nonstereoselective enzyme system [13–16]. Many researches have focused on the following two classes. One is the decolorization of various dyes by a single fungal strain, and the other is the decolorization of a single dye by various fungal strains [17–24]. But there is not much research which encompasses all the following information describing the effects on decolorization related to two different reaction types (solid and the liquid cultivation), two major decolorization mechanisms (extracellular enzymes and biosorption) and two different methods for the analysis of decolorization.

Accordingly, the objective of this study was to investigate the decolorization of six commercial dyes by ten fungal strains. Subsequently, the decolorization ability of *F. trogii* ATCC 200800 towards various commercial dyes was determined by investigating (a) biodegradation by extracellular enzymes and (b) biosorption on the cell biomass as two major mechanisms of dye decolorization. In order to investigate the possibility of application for wide range of environmental conditions, the experiments were performed using both solid and liquid batch cultivation, and repeated-batch cultivation.

2. Materials and methods

2.1. Fungal strains and cultivation conditions

Ten fungal strains, which are known to be able to degrade various dyes [17,18,20,21,23–26], were compared by investigation of their abilities to decolorize six different dyes. They were *Trametes versicolor* ATCC 12679, *Pleurotus ostreatus* ATCC 34675, *P. ostreatus* ATCC 9427, *F. trogii* ATCC 200800, *Sclerotium rolfsii* ATCC 200224, *P. chrysosporium* KCCM 60256, *Aspergillus niger* KCCM 60317, *Neurospora crassa* KCCM 60456, *Chrysonilia crassa* KCTC 6124 and *P. chrysosporium* KCTC 6147. All fungal strains were grown on potato dextrose agar (PDA) plates at 28 °C for 7 days and were preserved at 4 °C.

The experiments were performed not only by cultivation on a solid phase but also by cultivation in liquid. All fungal strains were precultured and they were prepared for the small pieces (the disk size of 1 cm^2 mycelium) on PDA. In the case of the solid cultivation, the fungal strains were inoculated onto a yeast malt peptone glucose (YMPG) agar medium containing 100 mg/l of the dyes and incubated at 28 °C statically for 15 days. YMPG consists of the following components (g/l of distilled water): glucose, 10; malt extract, 10; peptone, 2; asparagine, 1; KH_2PO_4, 2; $MgSO_4{\cdot}7H_2O$, 1; and thiamine–HCl, 0.001 [27]. The decolorization ability of *F. trogii* ATCC 200800 was also investigated in liquid batch and repeated-batch experiments. In the case of the batch liquid cultivation, the inoculums for the decolorization experiments were prepared by growing the fungal strains in potato dextrose broth (PDB) in a controlled incubator (28 °C and 100 rpm). After 4 days of cultivation, the mycelia from flasks were homogenized using an Ace Homogenizer (Nissei, Tokyo, Japan) at 1500 rpm for 30 s. Eight millilitre of the homogenized samples (0.036 g, dry weight) were used as an inoculum in flasks which contained 80 ml of Kirk's basal salts (KBS) medium [19] and 100 mg/l of the dye. The pH was adjusted to 4.5 by using 2,2-dimethyl succinic acid. KBS medium consists of following components (per 1 l distilled water): glucose, 5 g; ammonium tartrate, 0.22 g; KH_2PO_4, 0.2 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; $CaCl_2$, 0.01 g; thiamine–HCl, 0.001 g, 10% tween solution, 10 ml; veratryl alcohol, 0.23 ml; trace element solution, 10 ml. Trace elements solution has the following compositions (g/l of distilled water): $CuSO_4{\cdot}7H_2O$, 0.08; H_2MoO_4, 0.05; $MnSO_4{\cdot}4H_2O$, 0.07; $ZnSO_4{\cdot}7H_2O$, 0.043 and $Fe_2(SO_4)_3$, 0.05. In the case of the repeated-batch cultivation, the dyes were added repeatedly into the flasks after the disappearance of over 80% of the original color. Cultivation flasks were incubated on a shaking incubator (B. Braun Biotech International, Melsungen, German) under aerobic condition (28 °C and 100 rpm).

2.2. Materials

Materials for microbial growth were purchased from Difco (Sparks, MD, USA) and the other chemicals from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA). Six commercial dyes were tested and they were either anthraquinone-based (reactive blue 19, reactive blue 49 and acid violet 43) and azo-based (reactive black 5 and reactive orange 16, acid black 52 (1:1 metal complex)) dyes.

2.3. Color measurement

The degree of decolorization was measured spectrophotometrically by using a UV/vis spectrophotometer (Bio-Tek Instruments, Milano, Italy). In order to confirm the decolorization, two methods for the measurement of absorbance, namely Abs_{max} (an absorbance at the maximum peak) and ∫Abs (an integral absorbance between 400 and 700 nm), were used at each time point. Abs_{max} was obtained as the absorbance at the maximum peak for each dye and ∫Abs was calculated as the integral absorbance peak in the wavelength range between 400 and 700 nm for each dye. In order to investigate the decolorization by a biosorption mechanism, the fungal strains were centrifuged and then were mixed with 0.1 M of NaOH solution, and the sample mixed by vortexing for 1 min and then ultrasonicated (Branson, CT, USA) at 42 kHz for 30 min [28]. The absorbance of the solution was measured by UV/vis spectrophotometer.

2.4. Assay of enzyme activities

Laccase (EC 1.10.3.2) activity was determined by the oxidation of 4.47 mM syringaldazine in 0.15 M McIlvaine buffer (pH 4.6). The reaction mixture (3 ml) consisted of 2.5 ml of 0.15 M McIlvaine buffer (pH 4.6) containing 0.5 ml of the culture filtrate. The reaction was initiated by adding 0.01 ml of 4.47 mM syringaldazine solution. The increase in absorbance was measured at 525 nm at 40 °C using ε_{525} of 65,000 M^{-1} cm^{-1} to calculate laccase activity. The results were expressed in a unit defined as μM of quinone formed from syringaldazine per minute [29]. Lignin peroxidase (LiP) (EC 1.11.1.14) activity was based on the color change by oxidation of 1 μM azure B under defined condition [30]. The reaction mixture contained

Table 1
The decolorization with 10 fungal strains on the solid cultivation for 15 days

Strains	Anthraquinone-based dyes			Azo-based dyes		
	Reactive blue 19	Reactive blue 49	Acid violet 43	Reactive black 5	Reactive orange 16	Acid black 52
T. versicolor ATCC 12679	+	+	+++	−	+	+
P. ostreatus ATCC 34675	+	+	++	−	−	+
P. ostreatus ATCC 9427	−	−	−	−	−	−
F. trogii ATCC 200800	+++	+++	+++	+++	+++	+++
S. rolfsii ATCC 200224	−	−	−	−	−	−
P. chrysosporium KCCM 60256	+	+++	+	+++	+	+
A. niger KCCM 60317	−	−	−	−	−	−
N. crassa KCCM 60456	−	+	−	+	−	+
C. crassa KCTC 6124	+	+	−	+	−	+
P. chrysosporium KCTC 6147	+	+	+	+	+	+

Plates were assessed visually following 15 days of incubation, unless otherwise specified; the extent of decolorization was as follows: complete decolorization within 10 days (+++), complete decolorization within 15 days (++), partial decolorization (+) and no decolorization (−).

1 ml of 125 mM sodium tartrate buffer (pH 3.0), 0.5 ml of 0.16 mM azure B, 0.5 ml of the culture filtrate and 0.5 ml of 2 mM hydrogen peroxide. The reaction was initiated at 24 °C by adding hydrogen peroxide. Manganese peroxidase (MnP) (EC 1.11.1.13) activity was based on the color change by oxidation of ABTS [diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. The increase in absorbance was measured at 420 nm at 24 °C using ε_{420} of 36,000 M^{-1} cm^{-1} to calculate manganese peroxidase activity [31]. The reaction mixture (1 ml) consisted of 0.5 ml of 50 mM sodium lactate buffer (pH 4.5) containing 0.2 mM $MnSO_4$, 0.08 mg/ml of ABTS and 0.5 ml of 0.1 mM H_2O_2. The reaction was initiated at 24 °C by the addition 0.01 ml of the culture filtrate.

3. Results and discussion

3.1. Selection of fungal strain on the solid cultivation

Ten fungal strains were compared to investigate their potential for decolorizing the six dyes by cultivation on a solid phase. The decolorization abilities of the fungal strains after incubation for 15 days are shown in Table 1. Among the six dyes tested in this study, the blue colored dyes were by far the most rapidly decolorized, followed by the black dyes. *A. niger* KCCM 60317 and *P. ostreatus* ATCC 9427 were not able to decolorize all six dyes. Only slow and incomplete decolorization was observed with *P. chrysosporium* KCTC 6147 and KCCM 60256. In contrast, *F. trogii* ATCC 200800 was able to completely decolorize all six dyes in 10 days. Decolorization was begun with the formation of clear zones the small amount of biosorption around the colonies, and complete decolorization was finally assessed as the total disappearance of color without visible biosorption to the biomass. These results indicate that the major decolorization mechanisms are able to classified two kinds related to biodegradation and biosorption of dyes. From these results, the extracellular enzymes from *F. trogii* ATCC 200800 appeared to be very efficient for the decolorization of six dyes. The decolorization reactions associated with the extracellular enzymes are also shown in the liquid cultivation experiments.

3.2. Decolorization in the batch liquid cultivation

Preliminary experiments in liquid media were performed to determine the decolorization ability of *F. trogii* ATCC 200800. It was found that a temperature of 28 °C gave the most rapid decolorization with agitation rates of 100 rpm (data not shown). The initial concentration of each dye was 100 mg/l and the fungus grew up to a cell concentration about 16 g/l during operation (date not shown). *F. trogii* ATCC 200800 showed profiles of rapid decolorization activity in the liquid cultivation (Fig. 1). Whereas the complete decolorization by cultivation on the solid phase took 10 days, the decolorization in the liquid media required 3 days. In order to follow the decolorization by using UV/vis spectrophotometer, two different methods were adopted. One was the general method for the measurement of

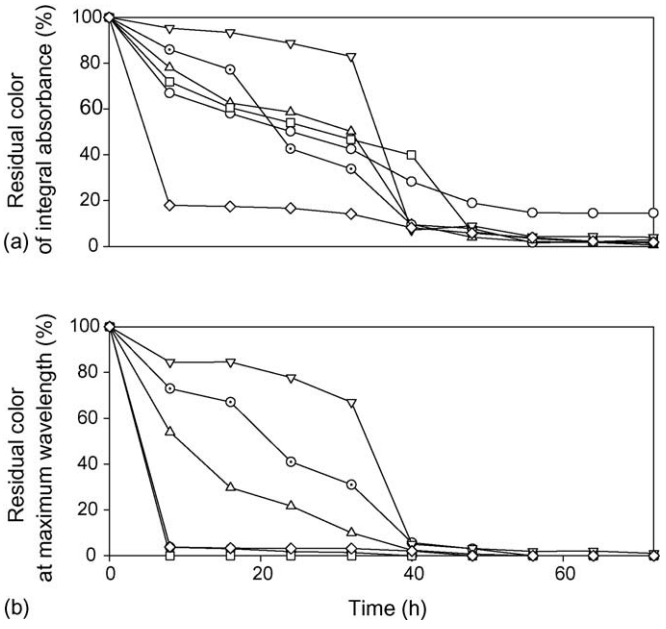

Fig. 1. The decolorization in liquid batch cultivation with *F. trogii*: (a) residual color by the method of ∫Abs and (b) residual color by the method of Abs_{max}. Symbols: reactive blue 19 (◯), reactive blue 49 (□), acid violet 43 (◊), reactive black 5 (△), reactive orange 16 (▽), acid black 52 (⊙)

Abs_{max} (Fig. 1(b)), the other was the specific method for the measurement of ∫Abs (Fig. 1(a)). The maximum wavelength of reactive blue 19, reactive blue 49, acid violet 43, reactive black 5, reactive orange 16 and acid black 52 were 594, 589, 574, 600, 568 and 496 nm, respectively. Each dye showed a different decolorization tendency and the anthraquinone-based dyes were decolorized faster than the azo-based dyes. In the case of reactive blue 19, reactive blue 49 and acid violet 43, they were decolorized 96.3, 100 and 96.2% within 8 h, respectively. Nearly complete decolorization (>98%) was achieved within 40 h. Reactive black 5, reactive orange 16 and acid black 52 were decolorized by 78.3, 22.2 and 59.0%, respectively, within 24 h. Nearly complete decolorization (>98%) of each of these three dyes was achieved within 56 h. Among the six dyes, reactive blue 49 was the most rapidly decolorized and reactive orange 16 showed the slowest decolorization rate. These results indicate that the small differences in dye structures, including steric effect and redox potential, could significantly affect the decolorization rate.

In order to investigate the complete decolorization, the degradation of the original dye and colored intermediates were measured by the integral absorbance (∫Abs) analysis at the wavelength range from 400 to 700 nm (Fig. 1(a)). All dyes were decolorized by over 96% within 56 h except reactive blue 19. Although significant decolorization of the six dyes determined by measurement of ∫Abs did occur, its extent was mostly less than that observed by measurement of Abs_{max}. The degree of decolorization by ∫Abs and Abs_{max} showed small differences due to the color of intermediates formed, but they showed similar trends of decolorization. In the case of anthraquinone-based dyes, the decolorization determined by measurement of Abs_{max} seemed to be faster than that of azo-based dyes. However, the rates of decrease in ∫Abs were smaller. This indicates that Abs_{max} decreased significantly, but the absorbance was increased by the presence of other colored intermediates. With azo-based dyes, ∫Abs and Abs_{max} decreased similarly. In addition, they show some different decolorization trends in Fig. 1(a and b). It was thought that the decolorization by the fungal strain should also take place the series of intermediates which generates color change similar to other researches [9,32]. The decolorization of azo-based dyes took longer than with the anthraquinone-based dyes. It has been known that degradation of azo dyes involves aromatic cleavage which has been found to be dependent on the identity of the ring substituents with the phenolic, amino, acetamido, 2-methoxyphenol or other easily biodegradable functional groups, resulting in a greater extent of degradation [33]. In particular, the phenomenon of color change also involves the rearrangement of identity, number and position of auxochrome [34]. An understanding of the biodegradation of molecules which occurred would require the chemical identification of the breakdown products, but such an analysis was not within the scope of the present work.

The production of extracellular enzymes was assayed in order to investigate the mechanism of decolorization by biodegradation. Under the experimental conditions in this study, extracellular laccase and MnP, but not LiP, were detected. These enzymes appear to be involved in decolorization and they are central to

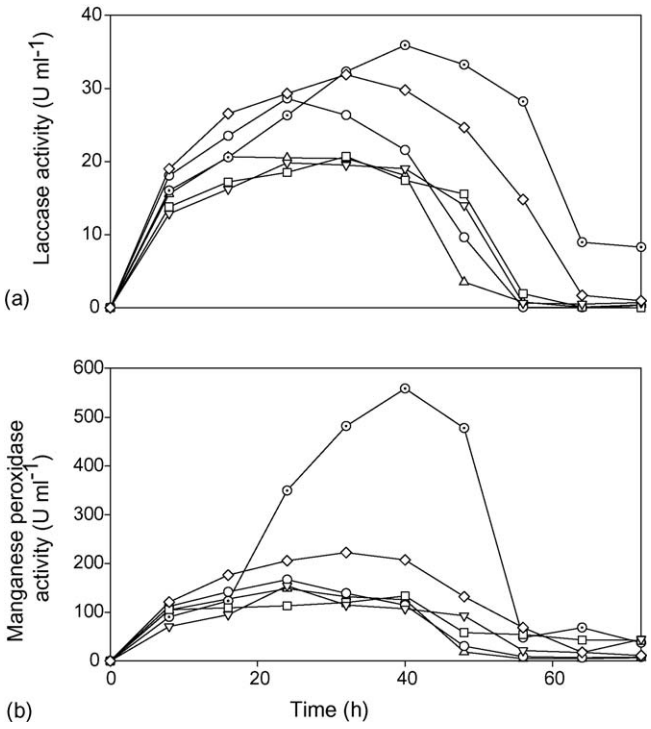

Fig. 2. Enzyme activity in liquid batch cultivation with *F. trogii*: (a) laccase activity and (b) manganese peroxidase activity. Symbols: reactive blue 19 (◯), reactive blue 49 (□), acid violet 43 (◊), reactive black 5 (△), reactive orange 16 (▽), acid black 52 (⊙)

F. trogii decolorization. The activities of laccase and MnP are show in Fig. 2. Broad peaks of MnP activity were typically seen from 8 to 48 h. Laccase also showed trends similar to those of MnP, but the activities were relatively low. The levels of laccase and MnP depended on the being degraded and these dyes, two enzymes showed the highest activities when acid black 52 was decolorized. In the case of the decolorization of acid black 52, laccase and MnP activities also occurred in culture supernatants, coinciding with the dye decolorization, with a maximum of 35.9 and 559.0 U/ml at 40 h, respectively. These results indicate that laccase and MnP from the *F. trogii* ATCC 200800 were important enzymes for the decolorization, and that decolorization is not a single step reaction but rather, a more complex phenomenon in which more than one enzyme is involved. The presence of laccase and MnP activities agreed with the findings obtained by other researchers that laccase-MnP combination is the most common group of extracellular enzymes in the white rot fungi [35,36]. It has also been proposed that the activity of laccase and/or MnP may be sufficient for lignin degradation in some fungi [35]. In addition, laccase is known as a multicopper enzyme that catalyzes the oxidation of phenolic and nonphenolic compounds [37]. MnP oxidizes Mn (II) to Mn (III), which is responsible for the oxidation of many phenolic compounds [38]. This simple mechanism of enzymatic decolorization would be valuable to real wastewater treatment if the mechanism and versatility of laccase-catalyzed and MnP-catalyzed decolorization of synthetic dyes could be understood.

Two major decolorization mechanisms by *F. trogii* ATCC 200800 are shown in Table 2. These results indicate that the

Table 2
The decolorization mechanisms with *F. trogii* in the liquid batch cultivation for 72 h

Time (h)	Anthraquinone-based dyes									Azo-based dyes								
	Reactive blue 19			Reactive blue 49			Acid violet 43			Reactive black 5			Reactive orange 16			Acid black 52		
	BS[a]	BD[b]	DC[c]	BS	BD	DC	BS	BD	DC	BS	BD	DC	BS	BD	DC	BS	BD	DC
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	96.3	96.3	0	100	100	3.5	92.7	96.2	8.0	38.0	46.0	5.6	9.9	15.5	17.9	9.1	27.0
16	0	97.0	97.0	0	100	100	5.0	91.7	96.7	6.9	63.4	70.3	6.0	10.4	15.4	24.3	8.6	32.9
24	11.7	86.5	98.2	0	100	100	8.8	88.1	96.9	6.4	71.9	78.3	7.2	15.0	22.2	40.5	18.5	59.0
32	10.7	87.9	98.6	0	100	100	8.0	88.9	96.9	0	90.0	90.0	9.3	23.7	33.0	33.5	35.5	69.0
40	0	100	100	0	100	100	7.5	90.5	98.0	0	97.6	97.6	10.0	85.0	95.0	24.1	70.2	94.3
48	0	100	100	0	100	100	0	100	100	0	99.3	99.3	12.3	84.6	96.9	20.0	77.0	97.0
56	0	100	100	0	100	100	0	100	100	0	100	100	2.1	96.0	98.1	1.1	98.9	100
64	0	100	100	0	100	100	0	100	100	0	100	100	0.8	97.2	98.0	0	100	100
72	0	100	100	0	100	100	0	100	100	0	100	100	0	99.0	99.0	0	100	100

[a] Biosorption.
[b] Biodegradation.
[c] Decolorization.

Table 3
The decolorization of six dyes with *F. trogii* in the repeated-batch cultivation for 5 days

Parameters	Anthraquinone-based dyes						Azo-based dyes					
	Reactive blue 19		Reactive blue 49		Acid violet 43		Reactive black 5		Reactive orange 16		Acid black 52	
Number of injection cycle	8	9	8	9	8	9	3	4	3	4	3	4
Dye injected (mg/l)	8 × 100	9 × 100	8 × 100	9 × 100	8 × 100	9 × 100	3 × 100	4 × 100	3 × 100	4 × 100	3 × 100	4 × 100
Dye removed (mg/l)	714.3	801.4	798.0	894.0	775.8	865.3	294.9	380.5	288.7	381.1	257.9	325.7
Decolorization (%)	89.3	89.0	99.8	99.3	97.0	96.1	98.3	95.1	96.2	95.3	86.0	81.4

major mechanisms could consist of biodegradation and biosorption. All dyes except reactive blue 49 were observed to be biosorbed to some extent. Reactive blue 19, acid violet 43, reactive black 5 and reactive orange 16 were biosorbed on *F. trogii* ATCC 200800 by about 10%, and acid black 52 was biosorbed by about 40%. Subsequently, the dyes, which were biosorbed on cell, came out to the culture media, which resulted in the increase of dye concentration in the culture media for a short period similar to the formational phenomenon of intermediates. These results clearly indicate that the decolorization by *F. trogii* ATCC 200800 involves complex mechanisms such as a combination of biodegradation by extracellular enzymes and biosorption by cell. The biodegradation was more important than biosorption for decolorization because *F. trogii* ATCC 200800 was able to produce the laccase or MnP to mineralize synthetic dyes [39,40]. However, the relative contributions of laccase and MnP to the decolorization of dyes may be different for each fungal strain and each dye [18]. In addition, the techniques by which decolorization occurs vary and among them adsorption seems to be promising for future development for bioremoval or biorecovery of dye substrates [6].

3.3. Decolorization in the repeated-batch cultivation

Repeated-batch experiments were performed to examine the longevity of the decolorization ability of *F. trogii* ATCC 200800 by successive additions of each dye (Table 3) to the batch system. Each dye (100 mg/l) was incubated with *F. trogii* ATCC 200800. After decolorization to an extent of over 80% had occurred, a further 100 mg/l of dye was added into the flasks. This process was repeated a number of times over a 5 days period. *F. trogii* ATCC 200800 sustained high rates of the decolorization following sequential additions of the different dyes. As the results, the dyes were removed in the range from approximately 300–900 mg/l within 5 days through the repeated-batch experiments. In the case of reactive blue 49 and acid black 52, 894 mg/l (99.3%) and 325 mg/l (81.4%) of dyes were removed in 5 days, respectively. The decolorization capability decreased as the number of successive additions increased during the repeated-batch operation. Anthraquinone-based dyes were also decolorized faster than azo-based dyes; these results paralleled the trends of the liquid batch experiments. These results show that the repeated-batch operations could decolorize the dye wastewater at a much faster rate in subsequent addition maintaining high decolorization activity (>80%). It indicated that this operation could reduce the toxic effect of high concentration of dyes. The eventual cessation of decolorization is likely to be due to nutrient depletion and the difference in the amount of dye that could be decolorized is a result of their different chemical structures.

4. Conclusion

This work evaluates ten fungal strains for their ability to decolorize six different dyes. Of them, *F. trogii* ATCC 200800

showed the greatest efficiency for decolorizing the six dyes when cultivated on a solid phase. The results of liquid-phase batch decolorization experiments showed that this organism's extracellular enzymes could efficiently decolorize all dyes. Anthraquinone-based dyes were completely decolorized within 8 h, whereas azo-based dyes required more than 40 h. The decolorization mechanisms by *F. trogii* ATCC 200800 involved a complex interaction of enzyme activity and biosorption. High decolorization was achieved over a period of 5 days during repeated-batch experiments. This study suggests that it is possible to decolorize high concentrations of commercial dyes, which would represent a significant advance in the treatment of wastewaters containing dyes. One of the most important factors that affect fungal decolorization is that dye molecules have many different chemical structures. Further research is needed to establish the relationships between dye molecule structure and fungal decolorization, and more studies are needed to develop a practical application.

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